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(54) Title: METHOD FOR THE RECOVERY OF PROTEINS BY PRECIPITATION

(57) Abstract: A method for recovery of a protein from a solution containing dissolved proteins by treating said solution with a chaotropic salt, which comprises - adding the chaotropic salt to the protein containing solution in an amount which is effective to precipitate the protein; and - recovering the protein as a precipitate.

METHOD FOR THE RECOVERY OF PROTEINS BY PRECIPITATION

Field of the invention

5 The present invention relates to a method for purification or separation of a protein from a solution containing dissolved proteins.

Background of the invention

10 Proteins that are used in the food industry are most often produced fermentatively in a microbial culture. These fermentatively produced proteins are in most cases used as a raw enzyme extract, i.e. in unpurified form, since the costs of protein purification are often too high. Following fermentation, the fermentation broth may be filtered, with the aim to separate the cells from the proteins, eliminate some of the small molecules in solution and concentrate the macromolecules such as the proteins. The extract thus obtained comprises the desired protein, and additionally other proteins and
15 macromolecules (polyphenols, colorants, etc.), which are produced by the cells or which have been introduced into the culture medium. The raw enzyme extract therefore has a very undefined composition due to its complexity.

Due to an increasing concern regarding the quality of food products and food additives, there is a need to eliminate any potential risk associated with secondary
20 metabolites which may be introduced by adding undefined compositions, such as raw protein extracts, to food products. It is therefore desirable to introduce a simple but effective purification step to recover the desired protein in a more purified form.

Proteins may be recovered from a protein containing solution by precipitation in a crystalline form. A large amount of literature, wherein the crystallisation of proteins is described, is available. EP 0 506 866 discloses a method for the crystallisation of
25 proteins wherein a crystallisation agent, such as Na, K, Ca or Mg formate, acetate or nitrate is added. Another way to crystallise proteins in a protein-containing aqueous solution is the treatment of the solution with a water-miscible organic solvent (WO 97/34919) in an amount sufficient to bring about crystallization. However, the addition of
30 an organic solvent to recover a protein for use in the food industry is not desirable. US 4,659,667 discloses a process for the crystallisation of proteins using evaporation of water under vacuum. Nevertheless, industrial evaporation processes are commonly very expensive, and are thus less attractive for application in the food industry.

An alternative crystallisation process for the recovery of a protein from a protein-containing solution has been disclosed in US 6,066,481. Here the protein solution is treated with a salt containing a sulphur atom having an oxidation state less than 6, and recovering the protein in crystalline form.

Summary of the invention

Surprisingly it has now been found that a protein can be recovered and partially purified from a solution containing dissolved proteins by the method of the present invention, wherein a protein is recovered from a solution containing dissolved proteins by treating said solution with a chaotropic salt, which comprises

- adding the chaotropic salt to the protein containing solution in an amount which is effective to precipitate the protein; and preferably
- recovering the protein as a precipitate. The solution is preferably an aqueous solution. We have also found that by the method of the present invention it is also possible to recover and purify more than one protein in subsequent order.

Detailed description of the invention

Chaotropic salts are known to increase the solubility of non polar substances in water. They can also denature proteins because they have the ability to disrupt hydrophobic interactions.

For example I^- , ClO_4^- and SCN^- are known to improve the solubility of proteins.

It has been surprisingly found that proteins such as enzymes can be precipitated under the conditions of the present invention.

Chaotropic salts that may be used in the present invention are for example salts of perchlorate (ClO_4^-), thiocyanate (SCN^-), hydrogensulfate (HSO_4^-), dihydrogenphosphate ($H_2PO_4^-$), hydrogencarbonate (HCO_3^-), iodide (I^-), chloride (Cl^-), nitrate (NO_3^-), guanidinium chloride, urea or trichloroacetate. Sulphate, for example ammonium sulphate, is not according to our definition a chaotropic salt but an kosmotropic salt or lyotropic salt.

Preferably, the chaotropic salt is a thiocyanate (SCN^-) or perchlorate (ClO_4^-). Preferably, the thiocyanate salt is a sodium or a potassium salt.

The thiocyanate salt may be added in solution or in an anhydrous form in an amount effective to precipitate a protein from the solution containing dissolved proteins. Preferably, the final concentration of thiocyanate in the solution containing dissolved proteins is at least 0.01 M, more preferably at least 0.02 M, and most preferably at least 0.03 M and/or the final concentration of thiocyanate in the solution containing dissolved protein is preferably lower than 2M, more preferably lower than 1M and most preferably lower than 0.5M. For the other chaotropic salts also concentrations of between 0.01M and 2M are also generally used. The effect of thiocyanate to precipitate protein according to invention can be enhanced by the addition of suitable amounts of sulphate to increase the yield of precipitate.

The protein may precipitate as an amorphous solid or as a crystal. An amorphous solid is defined as a solid of which the protein molecules have a diffuse structure. A crystal is defined as a solid having a regularly repeating internal arrangement of its protein molecules. Both the amorphous form and the crystalline form of the protein may be present at the same time in the precipitated product. The method of the invention results in a precipitated protein which is more pure (in wt% dry matter) than the protein in the starting composition. In this way it becomes possible to purify a protein from for example a fermentation broth whereby the protein has preferably a purity of at least 80 wt%, more preferably of at least 90 wt% on dry matter. Before this precipitation step the fermentation broth can be pretreated, for example be filtered or centrifuged in order to separate biomass or other unwanted components from the broth.

The present invention also provides a method wherein at least two proteins are separately precipitated from the solution containing the at least two dissolved proteins. According to this embodiment conditions are chosen wherein in first instance the first protein is precipitated. Hereafter the conditions can be amended to precipitate the second protein. In general the first precipitate will be removed before the second protein is precipitated. In case the precipitates differ in physical parameters, the precipitates can be separated afterwards in a fraction containing the precipitate of the first protein and a fraction of the precipitate of the second fraction. For example difference in particle size, density of the crystals and re-dissolvability can be used to separate the proteins.

On a commercial scale the quick precipitation is preferred. Quick precipitation generally results in a combination of a crystalline and amorphous precipitate. The precipitate is in a form of a slurry containing the solid product. This slurry can be dried

after which the solids may be granulated. Also the slurry can be used as the binder containing liquid needed for granulation. Preferably the slurry contains a high concentration of protein which can be dissolved again in solvents like glycerol or sorbitol. These solvents can be used as the formulation liquid which stabilizes the product microbially as well as stabilizes the protein itself. This process saves a substantial amount of glycerol and sorbitol compared to other processes for example ultra filtration (UF), which require more water and yield a lower concentration of protein.

The aqueous solution containing dissolved proteins as defined in the method of the present invention may be any solution comprising more than one protein. Preferably, the solution containing dissolved proteins is obtained from a fermentation broth. Such a fermentation broth may be obtained by fermentation of a microorganism, for example a bacterium or a fungus in any suitable nutrient medium.

A protein that is recovered by the method of the present invention from a solution containing dissolved proteins may be a protein such as an enzyme which is used in any industry, for example the food, pharmaceutical, chemical, analytical or detergent industry. A solution containing dissolved proteins in this regard may be a fermentation broth, or a cell culture and can be used for the production of pharmaceutical products. By dissolved proteins is meant a solution containing the protein which is a clear or cloudy solution, whereby the clear solution is preferred, so the protein is preferably completely dissolved. Also the use of not completely dissolved protein, such as a suspension, or protein dissolved using a dissolving improving agent such as a detergent are part of the present process.

In one embodiment of the method of present invention, the precipitated protein is an enzyme. The enzyme may be any enzyme used in the food, agricultural, pharmaceutical or detergent industry, such as a protease, a lipase, a pectinase, an amylase, a phytase, a cellulase, or a xylanase. Suitable enzymes to be precipitated according to method of the present invention include any enzyme that may be a fermentation product from a cell such as a microorganism. Preferably, the enzyme is from microbial origin, more preferably the enzyme is from fungal origin. Chemically or genetically modified mutants are included.

In a preferred embodiment, the enzyme that is precipitated according to the method of the present invention is an acid enzyme. Preferably, the pH at which the activity of the enzyme is optimal and the pH at which the enzyme is stable ranges between pH 1 and 7, more preferably between pH 2 and 5.

Examples of proteases to be precipitated according to the method of the present invention are acid proteases, such as Fromase®, an enzyme extract produced by DSM, Marzyme® produced by Rhodia, Microlant™ produced by Chr. Hansen, and Valiren® produced by Valley Research. These commercial preparations comprising an acid protease, which are all produced by *Rhizomucor miehei*, are used in the cheese-making industry for coagulation of milk. The *Rhizomucor miehei* protein extract may comprise, besides an acid protease, a β -glucanase, an esterase-lipase, and sometimes amylase as side activities. Another acid protease is chymosin which can be produced fermentatively in for example *K. lactis* or *A. niger*.

In a preferred embodiment of the present invention, precipitation occurs at a pH below the isoelectric point of the protein to be precipitated. Preferably, the precipitation occurs at a pH between 1 and 4, more preferably between 1.5 and 3.8.

The pH of the solution containing dissolved proteins in which precipitation of a protein occurs may be adjusted to the desired pH by the addition of any suitable acid or buffer solution. A suitable acid may be phosphoric acid or hydrochloric acid. A suitable buffer solution may be a phosphate-citrate buffer solution.

The temperature at which precipitation of a protein in a solution containing dissolved proteins occurs is between 2°C and 50°C. Preferably, said temperature is between 3°C and 30°C.

The protein precipitated by the method of the current invention can be recovered by standard methods known to a person skilled in the art.

The following examples are included for illustrative purposes only and are not to be construed as being limitative to the invention.

25

EXAMPLES

Definition of the yields

Yield based on mass

$$Y_m = (\text{mass of recovered protein}) / (\text{mass of initial protein}) * 100 (\%)$$

The protein content of the starting sample and end product sample is determined (Lowry). An electrophoresis on agarose gel (Sebia gel HR) is then performed on both samples in order to determine the ratio of the different proteins present in the solution. From this ratio, the mass of each protein (for example enzyme) can be calculated.

Yield based on enzyme activity

$$Y_e = (\text{total activity of recovered enzyme}) / (\text{total activity of initial enzyme}) * 100 (\%)$$

5

Example 1**Precipitation of Chymosin from *Kluyveromyces lactis* in the presence of sodium thiocyanate**

A solution is made containing a commercially available chymosin (Maxiren®, 8 g/l chymosin) and 0.04M sodium thiocyanate. This solution is kept at pH 2.5 and 5°C.

10

Within less than 24 hours, precipitation occurred. Subsequently the precipitate was centrifuged and lyophilized and analysed for enzymatic activity. The yield (Y_e) of chymosin is 90%. The purity was increased from 50% (starting solution) to 90% of the precipitate (based on total protein content wt%).

15

Example 2

Crystallisation of endo-1,3 (4) β -glucanase from *Rhizomucor miehei* in the presence of sodium thiocyanate

A solution is made containing 20-25% W/W endo-1,3 (4)- β -glucanase of the total protein content and containing sodium thiocyanate within the range 0-0.15M is kept at pH 3.0 and 5°C. Within less than 24 hours, crystals of the pure enzyme (99% w/w of total protein) are obtained Subsequently, the crystals were centrifuged and lyophilised. The crystals have a needle morphology of a 100 μ m size, as determined by transmission electronic microscopy.

The activity of endo-1,3 (4) β -glucanase determined as follows: 2 ml of the enzyme solution was added to 15 ml of a 1% (v/v) solution of barley cream (Moulin Waast, Mons en Pevele) at pH 5.6 and at 45°C. The decrease in viscosity was determined in Hubbelhode no° 1 C viscosimeter, during 15 min. One unit of BGF (endo-1,3 (4)- β -glucanase) is defined as the enzymatic activity which will lead to a variation of viscosity with a speed which constant is 0.147 min⁻¹ for 1 ml of medium in the assay conditions.

Table 1 shows the Y_m and Y_e as a function of the initial thiocyanate concentration and as a function of the initial protein concentration.

Table 1. Y_m / Y_e (%) as a function of the sodium thiocyanate concentration

Initial protein concentration (g/l)	Sodium thiocyanate concentration (M)	Y_m (%)	Y_e (%)
16	0	1.6	
	0.04	66.0	
	0.05	69.7	
	0.06	53.3	
	0.07	46.3	
26	0	1.6	
	0.02	84.6	
	0.05	83.9	
	0.06	86.9	
	0.17	78.1	87.1

Table 1 shows that both the initial protein concentration and the initial thiocyanate concentration have an effect on the yields. Yield higher than 80% can be achieved.

5 Table 2 shows the effect of the pH on the crystallisation of endo-1,3 (4)- β -glucanase. pH stability range of the enzyme (pH range 2.5-7.5) with a fixed sodium thiocyanate concentration of 0.04M (protein concentration 16 g/l).

Table 2. Effect of pH on crystallisation

pH	Y_m (%)	Y_e (%)	Morphology
2.0	47.5	43.0	Needles
2.5	71.8		Needles
3.0	73.4		Needles
3.5	34.0		Needles
4	1.7		Needles
4.5	0		No crystal
5	0		No crystal
6	0		No crystal
7.5	0		No crystal

10 Table 2 shows that no crystals are formed above pH 4. The crystals are only formed below the isoelectric point of enzyme (pH 4.2).

Both tables show that there is no denaturation of the enzyme (Y_m is comparable to Y_e).

Example 3

15 Crystallisation of endo-1,3 (4) β -glucanase from *Rhizomucor miehei* in the presence of sodium perchlorate

A solution was containing 30-37.5 g/l endo-1,3 (4) β -glucanase from *Rhizomucor miehei* and containing 0.04 M of sodium perchlorate was kept at pH 3 and 5°C. The endoglucanase was added as Fromase and therefore protease was also present. Within
 20 less than 24 hours, protein crystals (needle shape) were obtained. The Y_m was 72% and the Y_e was 73%, which indicated that no denaturation of the enzyme had occurred.

Example 4

Effect of sulfate (SO_4^{2-}) on the crystallisation with sodium thiocyanate of endo-1,3 (4) β -glucanase from *Rhizomucor miehei* .

5 A solution was containing 9 g/l endo-1,3 (4) β -glucanase from *Rhizomucor miehei* and containing 0.04 M of sodium perchlorate was kept at pH 3 and 5°C. Ammonium sulfate was added to a concentration range from 0 to 2M. Within less than 24 hours, crystals of the pure enzyme were obtained. Results are shown in Table 3.

10 **Table 3.** Effect of the addition of sulfate on the Y_m of the crystallisation of endo-1,3 (4)- β -glucanase (9 g/l)

Ammonium sulfate (M)	0	1.25	1.75	2
Y_m (%)	28.5	64.0	75.5	86.1

15 Table 3 shows that an increased sulfate concentration (0-2 M) results in an increase of the Y_m of the enzyme crystallisation process with sodium thiocyanate.

Example 5

Precipitation of protease from *Rhizomucor miehei*

20 A solution is made containing Fromase® (containing both β -glucanase and protease), protease being present in the solution 60-67.5 g/l, and thiocyanate (0.04M). The solution is incubated for one day at pH 3 and 5°C. The β -glucanase is first recovered. Then the thiocyanate concentration is increased to 0.1M. This solution is kept at pH 3 and 5°C. After less than 24 hours of incubation, precipitation occurs. The Y_m and the Y_e were both 19%, which indicated that no denaturation of the protein had occurred. The example shows that the separation of two enzymes is possible.

25 The activity of protease was measured as described by the International Dairy Federation (IDF); protocol 157:1992 and can be expressed in IMCU (International Milk Clotting Unit).

When the thiocyanate concentration is 0.17 M, the Y_m was increased to 25%, whereas the Y_o was decreased to 16%. The protease in the Fromase®, may have been denatured at this higher thiocyanate-concentration.

CLAIMS

1. A method for purification or separation of a protein from a solution containing dissolved proteins comprising
 - 5 - adding a chaotropic salt to the solution containing dissolved proteins in an amount which is effective to precipitate the protein whereby the precipitated protein is more pure than the dissolved protein (wt% dry matter); and preferably
 - recovering the protein as a precipitate.
2. A method according to claim 1, wherein the precipitate is in an amorphous or
10 crystal form of the protein.
3. A method according to claim 1, wherein the precipitate is a mixture of both crystal and amorphous forms.
4. A method according to any one of claims 1 to 3, wherein the precipitate is further dissolved in glycerol or sorbitol.
- 15 5. A method according to claim 1, wherein precipitation occurs at a pH below the isoelectric point of the protein to be precipitated.
6. A method according to anyone of the preceding claims, wherein precipitation occurs at a pH of from 1 to 4, preferably at a pH of from 1.5 to 3.0, by the addition of one of the following:
 - 20 - an acid, preferably phosphoric acid or hydrochloric acid; and
 - a buffer solution, preferably a phosphate-citrate buffer solution.
7. A method according to any one of claims 1 to 6 wherein at least two proteins are separately precipitated from the solution containing the at least two dissolved proteins.
- 25 8. A method according to any of claims 1 to 7, wherein the chaotropic salt is a thiocyanate salt, preferably a sodium or potassium salt, or the chaotropic salt is a perchlorate.
9. A method according to claim 8, wherein the thiocyanate salt is added in solution or in an anhydrous form.
- 30 10. A method according to claim 8 or 9, wherein the final concentration of thiocyanate is at least 0.01M and/or lower than 2M.
11. A method according to any one of the preceding claims wherein sulphate is added to the solution containing dissolved proteins preferably the final

concentration of sulphate is at least 0.01M, more preferably at least 0.1M and/or preferably lower than 3M, and more preferably lower than 2M.

12. A method according to any one of the preceding claims wherein said protein in the solution containing dissolved proteins, is fermentatively produced.
- 5 13. A method according to claim 12, wherein the protein is fermentatively produced in *R. miehei*, *K. lactis* or *A.niger*.
14. A method according to any one of the preceding claims, wherein the protein is an enzyme.
- 10 15. A method according to any one of the preceding claims, wherein the enzyme is an acid enzyme.
16. A method according to claim 15, wherein the acid enzyme is an acid protease, preferably chymosin or rennin.
17. A method according to any one of the preceding claims, wherein the precipitation occurs at a temperature between 2 and 50°C.